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The Evolution of Energy-Transducing Systems. Studies with Archaebacteria.

Semiannual Progress Report, September 1996 - February 1997

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Summary

All known F-ATPases are inhibited by the binding of 1 Mol DCCD[2] per Mol of enzyme; the binding site is Glu 193 (*E. coli* numbering) of the β subunit. In earlier work it was shown that the membrane ATPase from *Halobacterium saccharovorum* is also inhibited by DCCD and that binding occurred to subunit II. However, cations or nucleotides did not offer protection against inhibition. From the published 3 D structure of the bovine F-ATPase (Abrahams et al.1994) it is known that the DCCD-binding Glu residue is located in the nucleotide binding site. Cyanogen bromide cleavage of halobacterial subunit II had indicated that DCCD bound to a region between amino acids 379 (Glu) to 442 (Met); assuming structural analogy to F-ATPases, this area is well away from the nucleotide binding site, but is located in the region of the "catch" between β and γ in bovine F-ATPase. We have repeatedly obtained evidence that the same region includes conserved epitopes, as judged from a crossreaction between antiserum angainst subunit II and cyanogen bromide fragments of the β subunit from *E. coli* F-ATPase. It was tried to improve the visualization of this crossreaction by using the new SuperSignal system (Pierce) for Western blotting, and to recycle protein-A-gold stained blots.
Work on the characterization of an extremely halophilic isolate (*Halococcus salifodinae*) from Permian salt sediments, which appears to be an autotroph, was continued with experimens directed to identify ^{14}C-labeled products by MALDI-TOF[2] mass spectrometry. These results have been submitted for publication.

[2]Abbreviations: DCCD, dicyclohexylcarbodiimide; CNBr, cyanogen bromide; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight.

Progress report

1. The ATPase from *H. saccharovorum* is inhibited completely by approx. 50 µM DCCD[2] when incubation takes place at acidic pH (Kristjansson and Hochstein 1985). This is quite similar to the reaction of DCCD with one of the β subunits of F-ATPases, which results in enzyme inactivation. The bulk of DCCD is incorporated into subunit II of the halobacterial ATPase. However, nucleotides did not protect against the inhibition by DCCD, nor did divalent cations, as is the case with F-ATPases. When subunit II from DCCD-labeled halobacterial ATPase was cleaved with CNBr[2], incorporation of the inhibitor was detected in two peptides of M_r 14 000 and 6000 (14K and 6K). These peptides were N-terminally sequenced; 11 amino acids of the 6K peptide were identical with residues starting from Ala 380 of the sequence from *H. halobium* (Ihara and Mukohata 1991). The 14K fragment, which also incorporated the label, is probably due to incomplete cleavage.

Although the exact position of the reactive amino acid (probably a glutamic acid) has not been determined, the data suggested conserved sequences which may be involved in subunit interactions. Support for this contention came from immuno reactions. Antisera against the two major subunits had been prepared in an earlier working period of this Cooperative Agreement. When the F_1-ATPase from *E. coli* was probed in Western blots with antiserum against subunit I, no crossreaction with any of the *E coli* subunits was found. However, antiserum against subunit II did react with both the α and β subunits of *E. coli*. To explore this results further and to determine, where exactly the crossreactions originated from, CNBr fragments of the α and β subunits from *E. coli* F-ATPase were prepared and examined in Western blots. There was only a weak immuno reaction left with the α fragments, but there were two fragments from the β subunit, which showed a strong reaction in the immunoblot. The molecular masses of these fragments were about 10K and 17K. N-terminal sequencing confirmed that they corresponded to the CNBr peptides starting at Asp 380 and Gly 276, respectively. These fragments started with or contained the sequence DELSEED, respectively; this is a very conserved sequence in all F-type ATPases from various organisms. From the paper of Abrahams et al.(1994), where the 2.8 Å resolution of crystals of the bovine F-ATPase was reported, it became

clear, that the DELSEED stretch is located on an edge of the β subunits, well away from the nucleotide binding sites, and that it makes contact with the γ subunit; this region has been termed the "catch" between β and γ subunits. Our results suggested that a disruption of cooperativity between the halobacterial subunits might take place upon reaction with the bulky reagent DCCD.

The standard procedure, which we use to visualize immuno complexes in Western blots, is the reaction with protein A-gold, which binds specifically to the Fab fragment of antibodies, and results ideally in a stable pink colour on a white background membrane (see Hochstein 1996). If necessary, this reaction may be enhanced by the formation of silver grains, whereby the colloidal gold of protein A provides the nucleation sites.

The colour contrast between immuno reactive fragments and background in our experiments was deemed to be too low for successful photography and eventual publication of the data. Therefore we switched to the new "SuperSignal system", by Pierce Co., for visualization of immuno complexes. In this procedure, horse radish peroxidase is coupled via an antibody to the immuno complexes, and a chemiluminescent signal is produced by the addition of peroxide-luminol buffer, which is detected on X-ray film. Although claimed to be highly sensitive, we found that the minimal amount of protein, which can be detected, is not greater than that which is seen with the protein-A-gold system. In addition, there were problems with high background signals; the reasons for this were apparently the solutions to be used for blocking non-reacting sites on the membranes. All solutions are supplied by the manufacturer, which does not leave much room for variation of experimental protocols. For the time being, we have returned to the protein-A-gold system for visualization. An improvement was made by using dried fragment-containing gels, which can be rehydrated and blotted. This allows a precise location of immuno reactive bands; in addition, each step in the procedure can be observed.

2. *Halococcus salifodinae* was isolated from Austrian rock salt which is believed to be deposited about 250 million years ago (Denner et al. 1994). A few halophilic archaebacteria are known to possess the enzyme ribulose biphosphate carboxylase; however, all of those appear to be

heterotrophs. Growth of *Hc. salifodinae* in a minimal medium in the absence of an added carbon source (containing only 0.02 % yeast extract) strongly suggested autotrophic growth. The incorporation of ^{14}C-labeled sodium bicarbonate into whole cells and cell free extracts of *Hc. salifodinae* was demonstrated; propionic acid and NH_4Cl stimulated this incorporation about 1.5 fold. Ribulose 1,5 diphosphate did not stimulate uptake, indicating that *Hc. salifodinae* does not possess the enzyme ribulose biphosphate carboxylase. Thin layer chromatography of lysed cells, following labeling, revealed one major radioactive spot. Its identification was attempted with mass spectrometry (MALDI-TOF); none of the amino acids or other small molecular weight metabolites, associated with known carbon assimilation pathways, appeared to match; rather, indication for the presence of a hexose-phosphate was found. These results, together with an updated phylogeny of halophilic archaebacteria, based on a complete 16S rRNA sequence of *Hc. salifodinae* , were submitted to the journal "Origins of Life and Evolution of the Biosphere (Glaser and Stan-Lotter).

References:

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Other Activities

September 1996 - February 1997: Teaching appointments at the University of Vienna and University of Salzburg, Austria, while remaining at reduced time (10%) with the SETI Institute.